

Cell Systems, Volume 4

Supplemental Information

Measuring Signaling and RNA-Seq in the Same

Cell Links Gene Expression to Dynamic

Patterns of NF- κ B Activation

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Figure S1

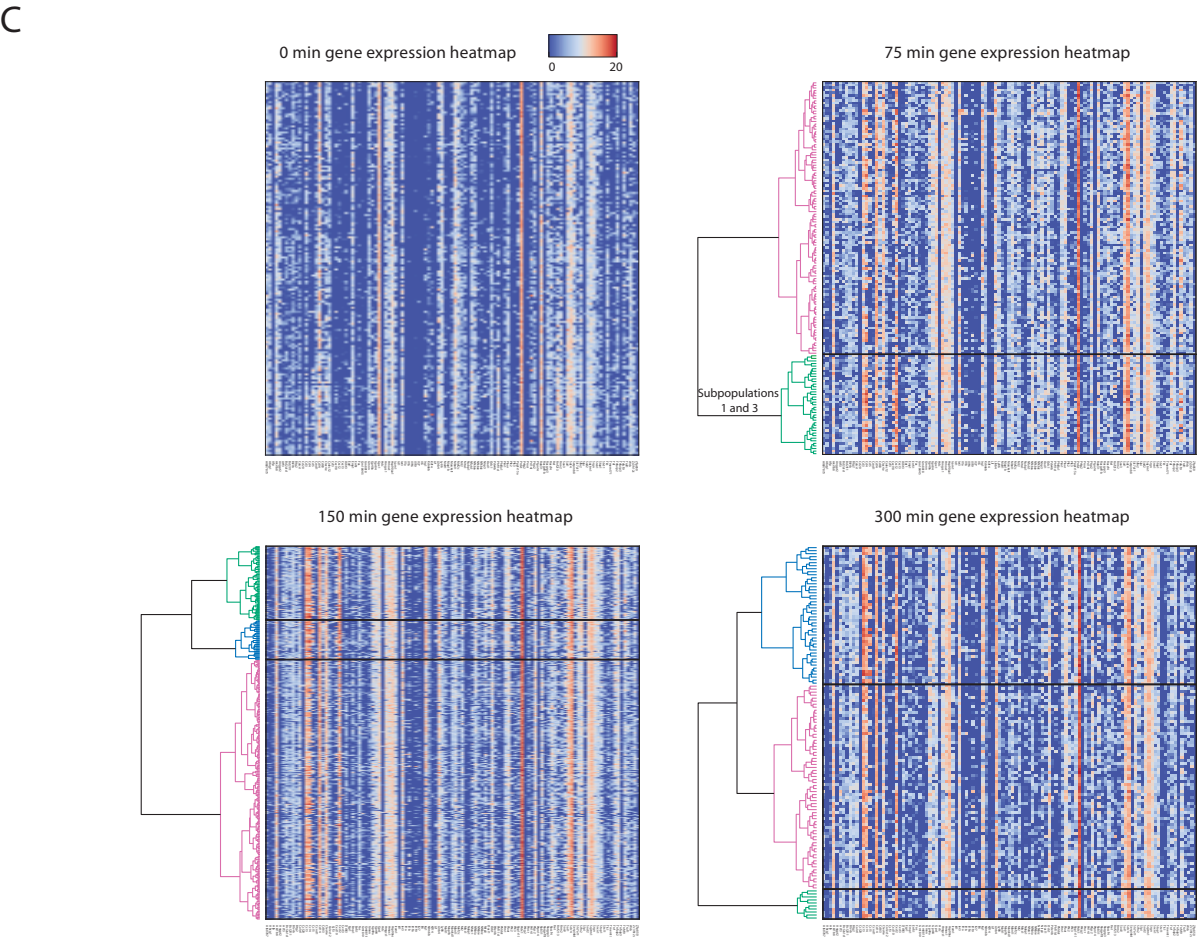
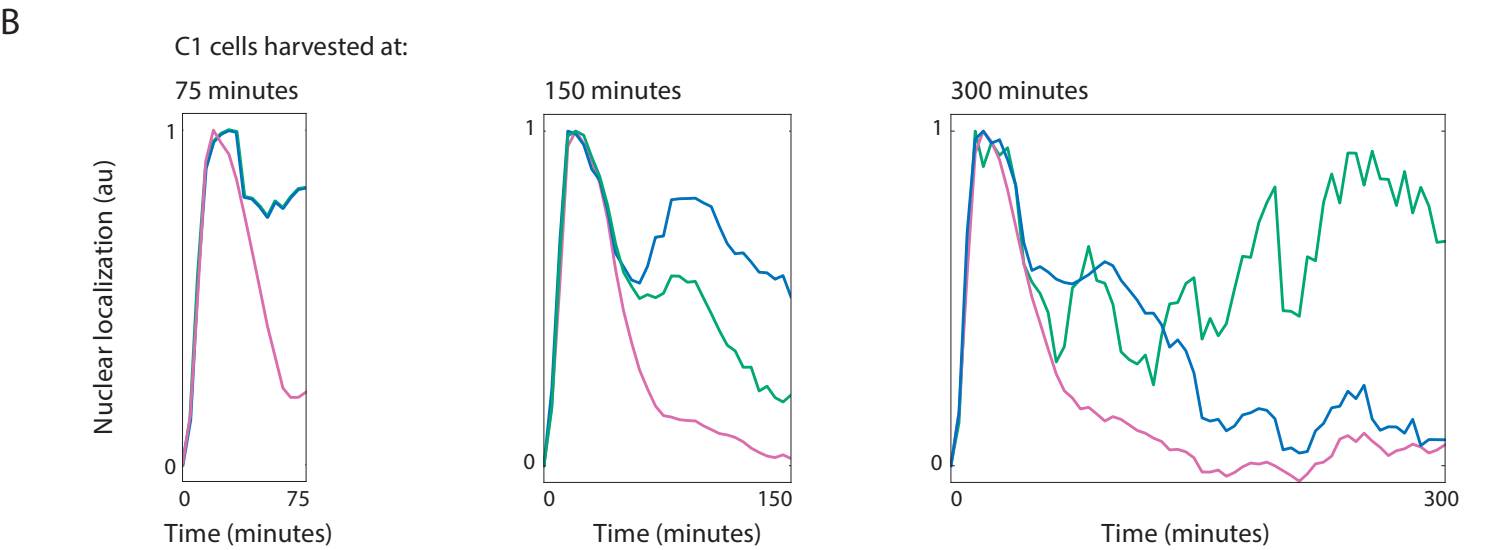
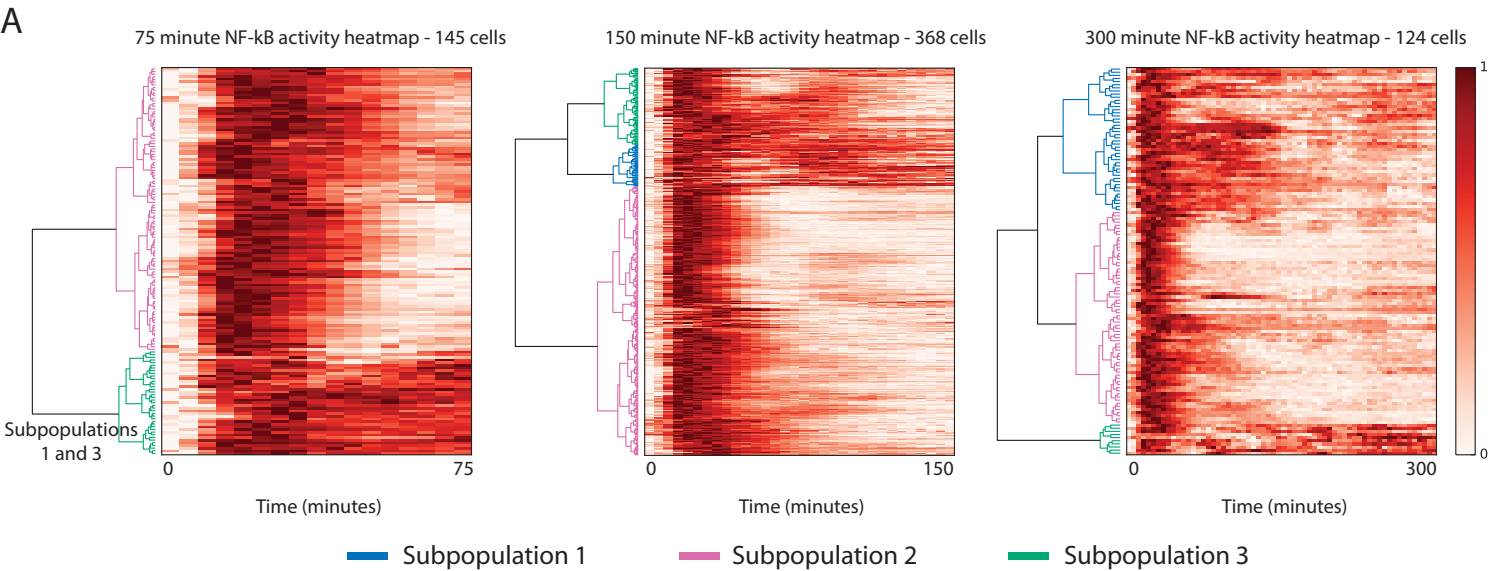
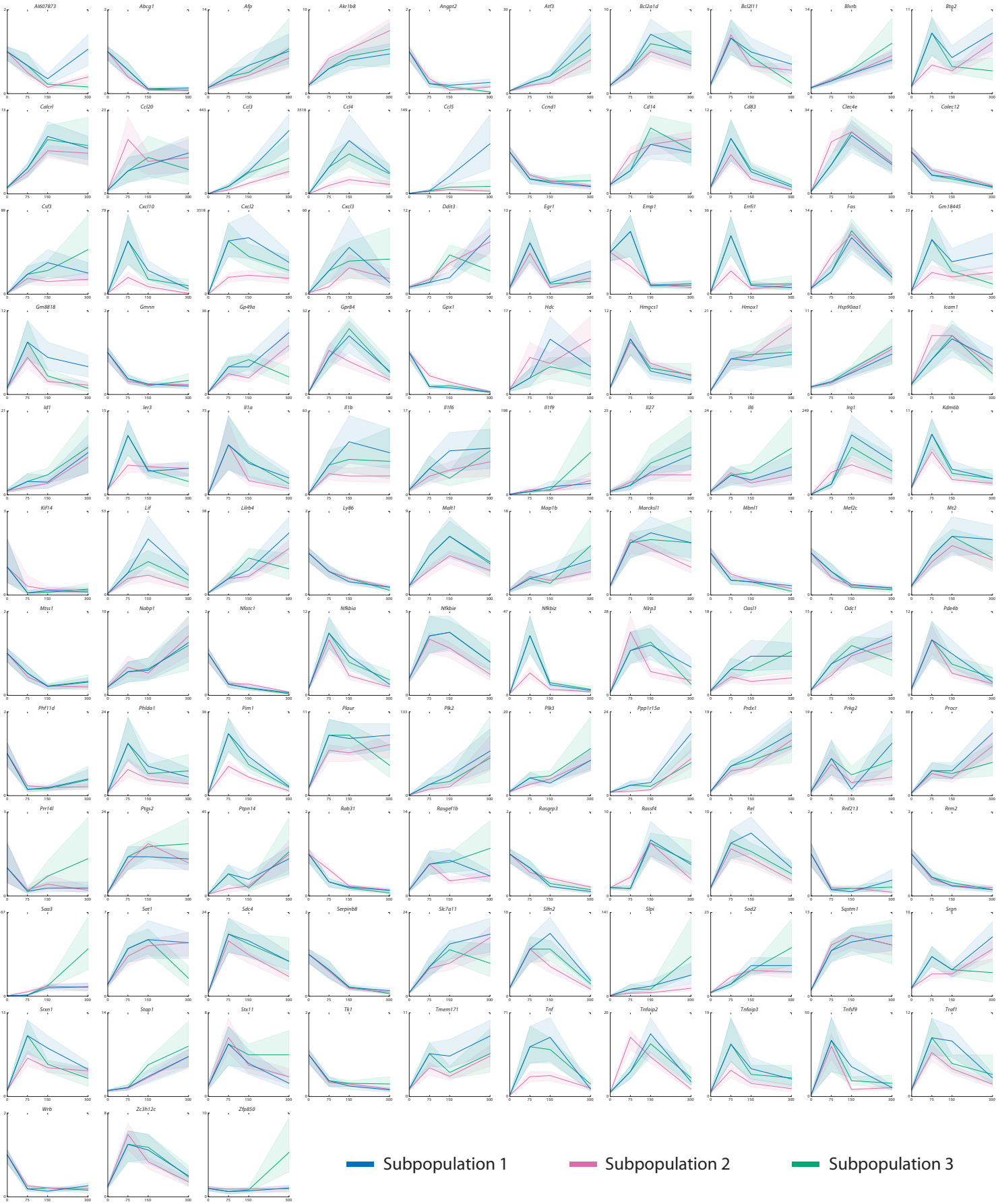


Figure S2

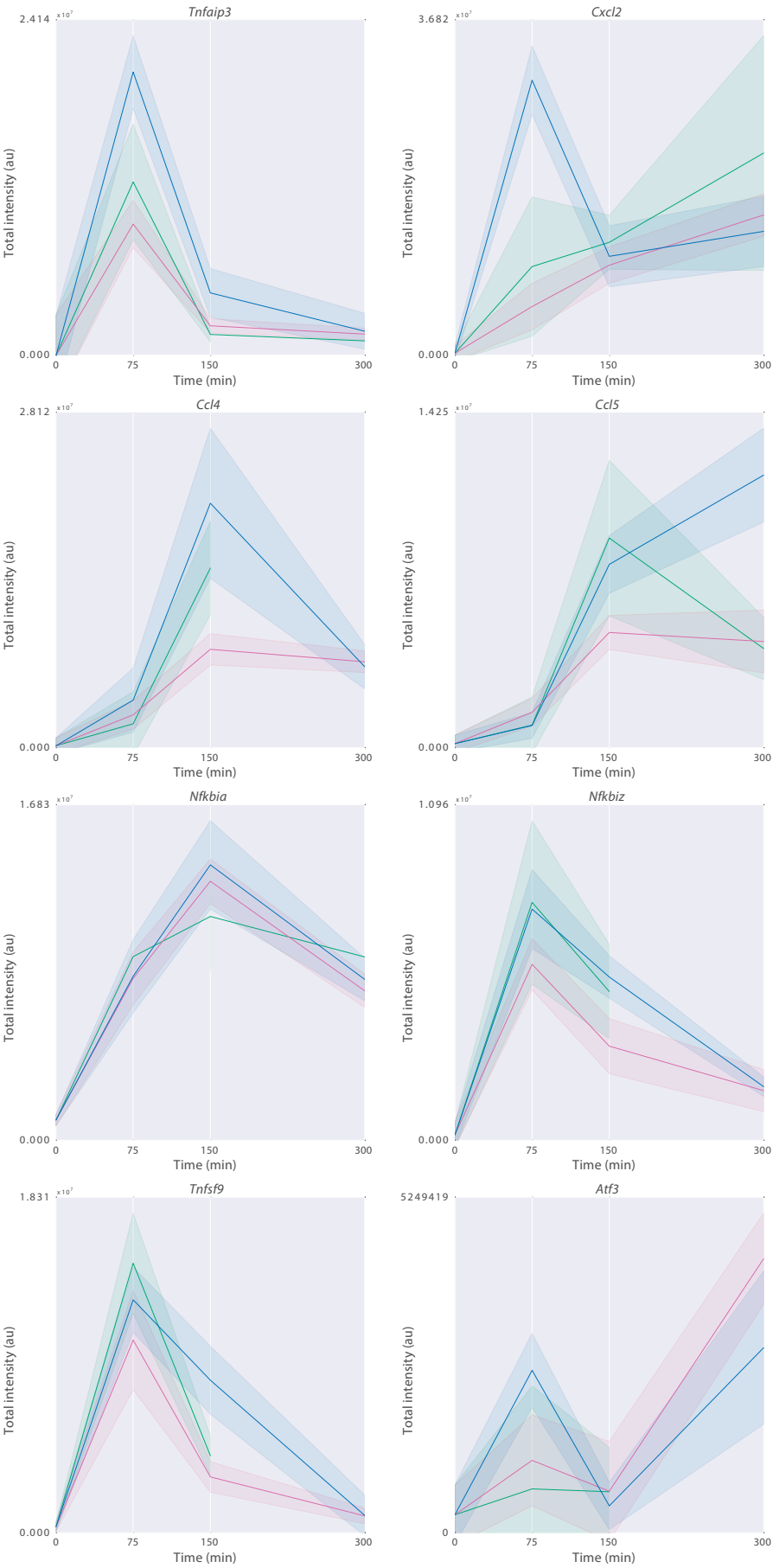
Fold change



Time (minutes)

Figure S3

A



B

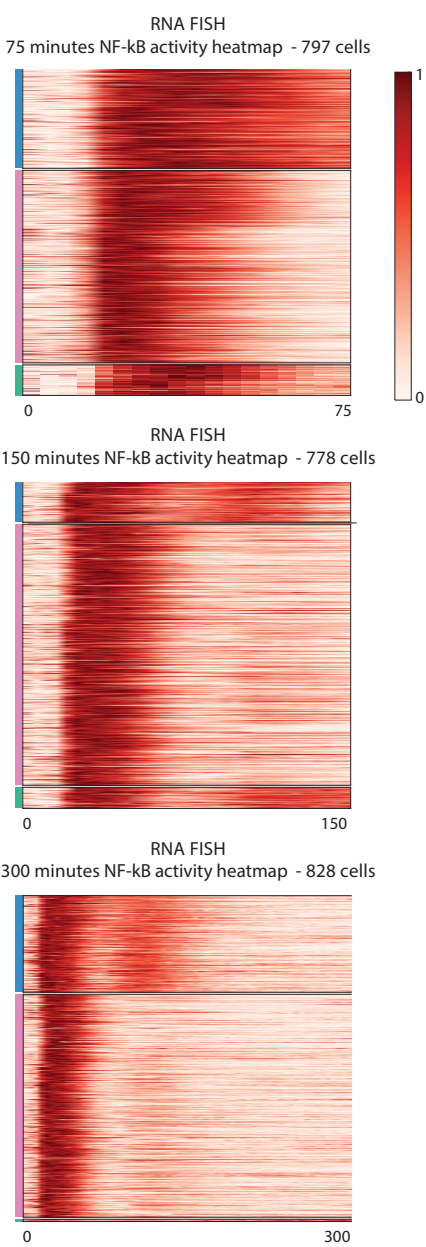


Figure S4

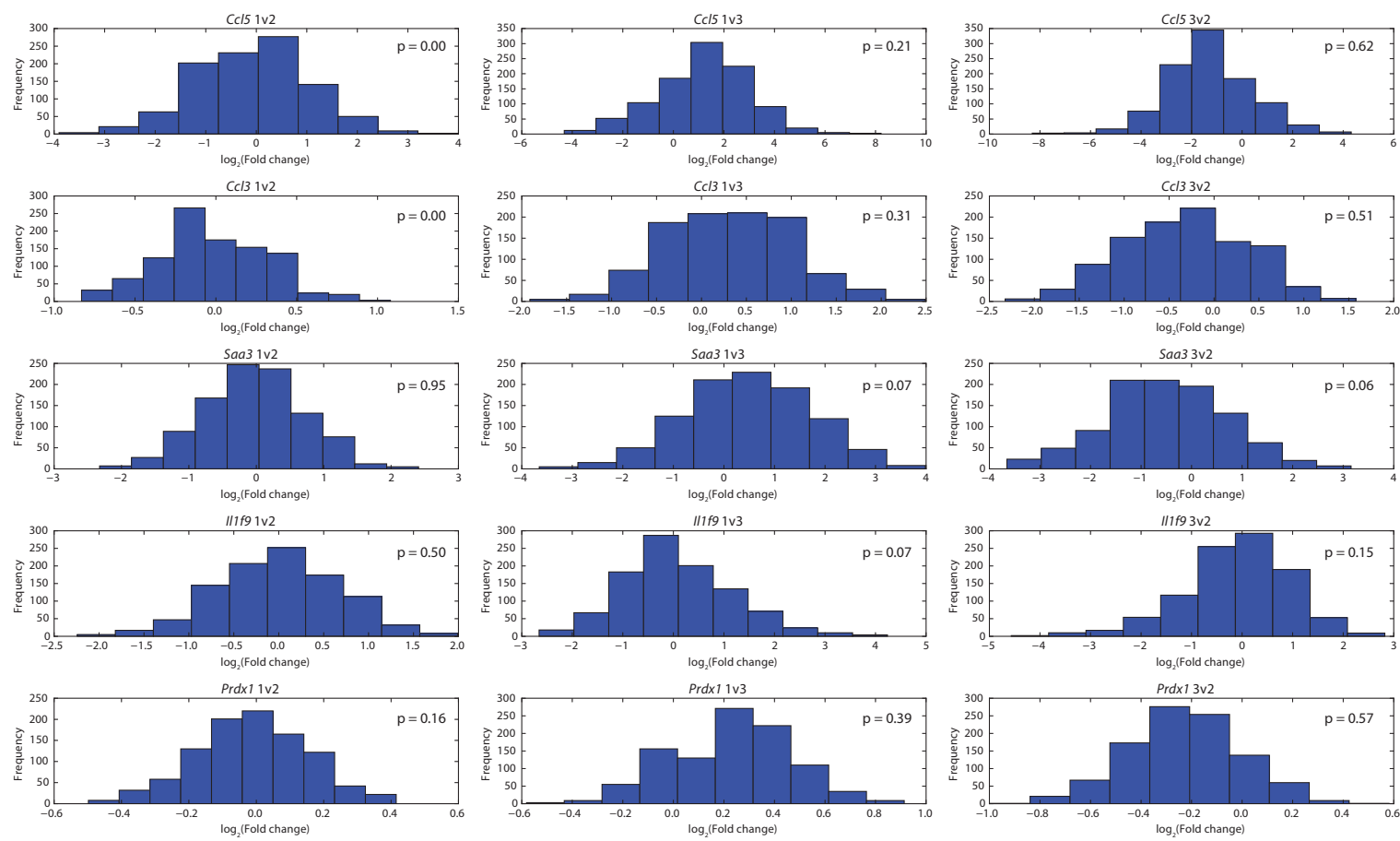
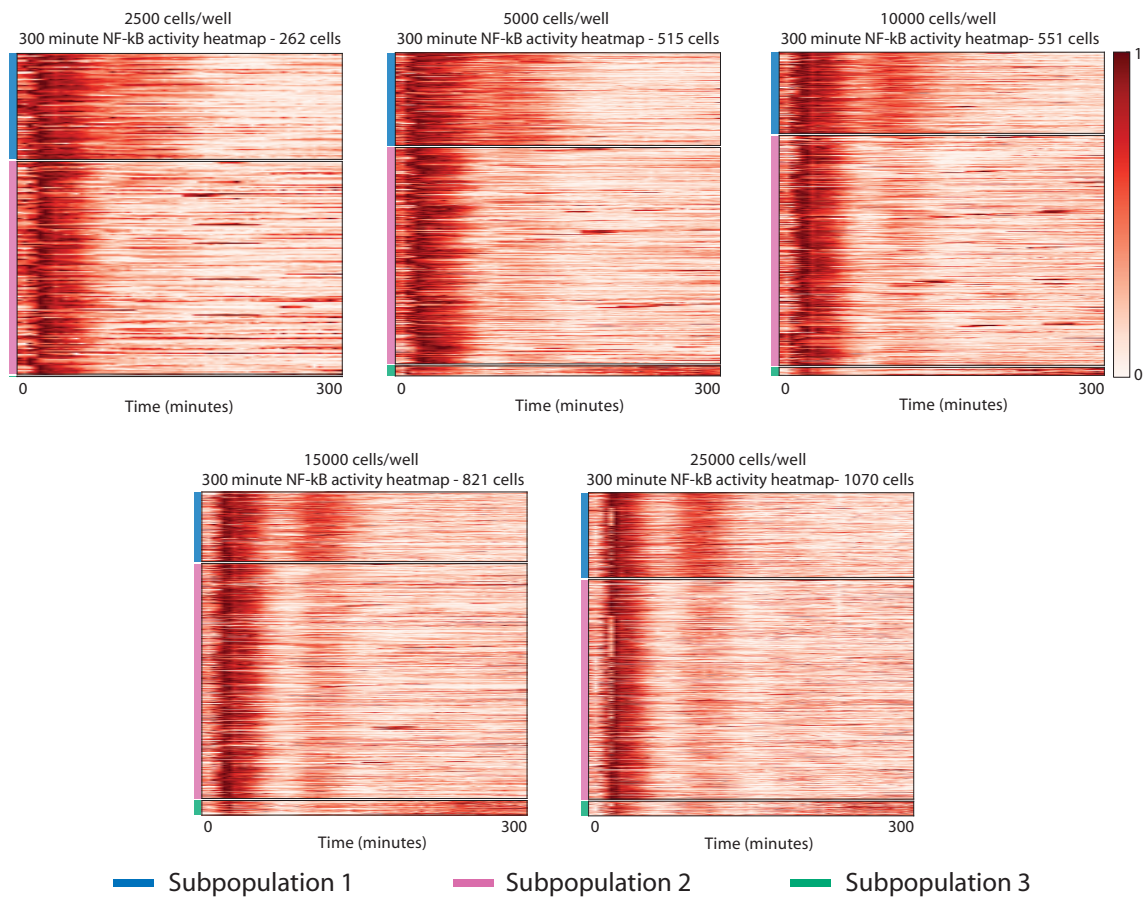
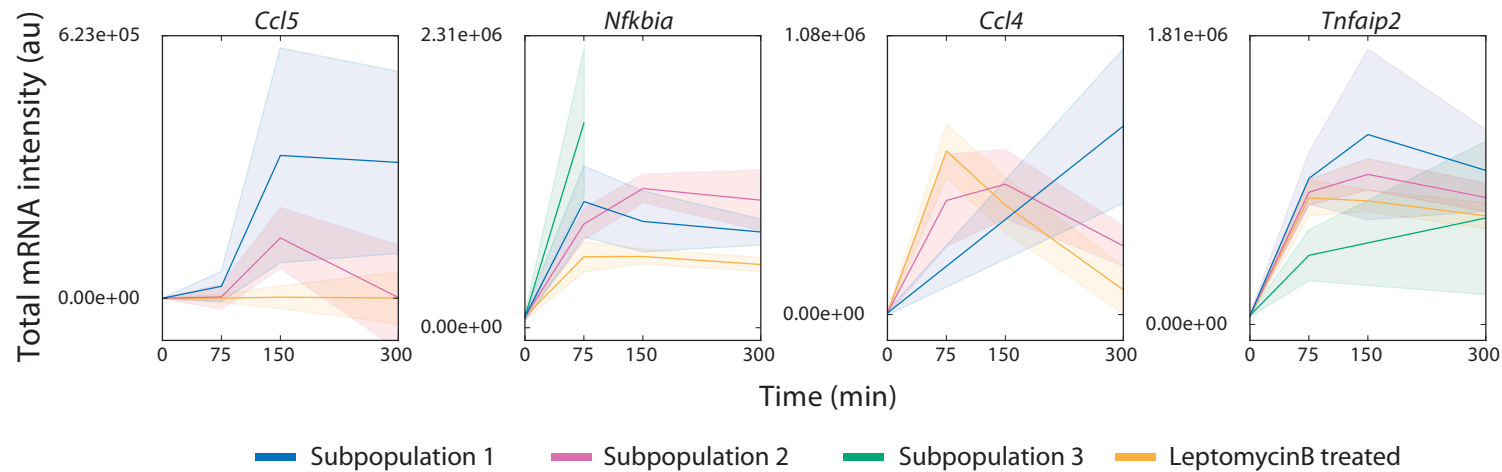


Figure S5

A



B



C

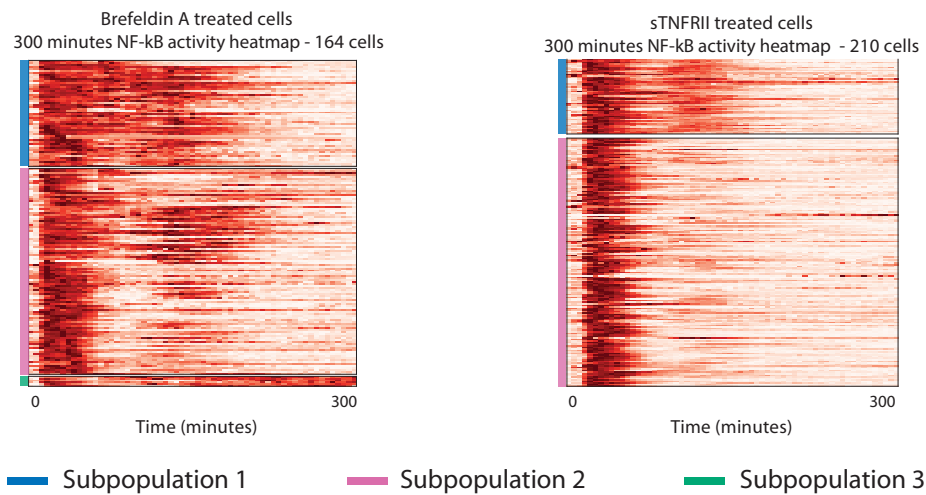


Figure S6

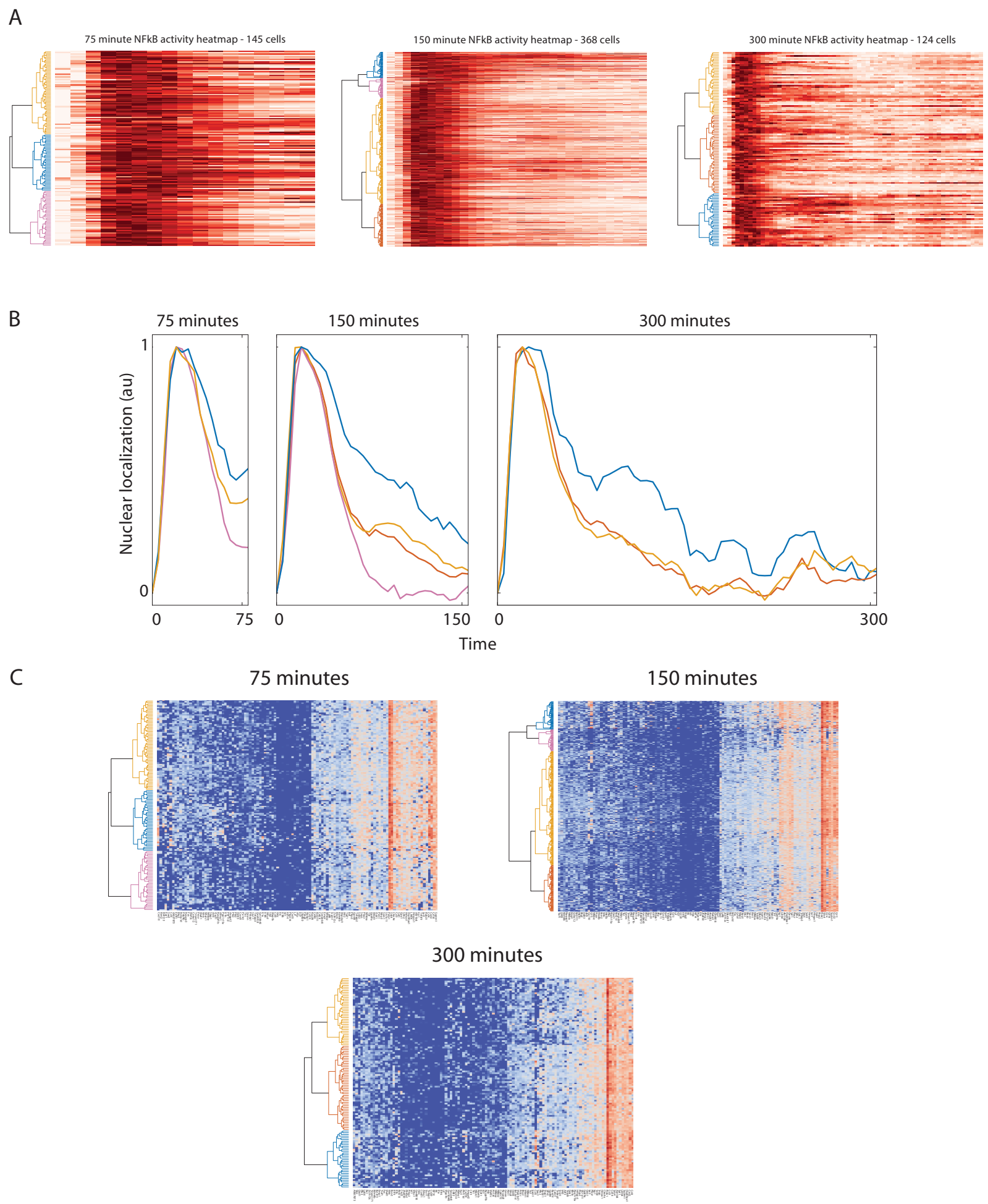
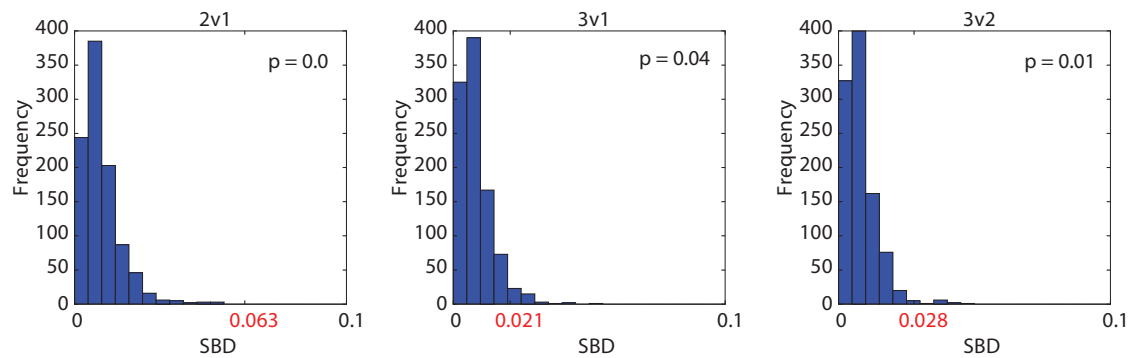
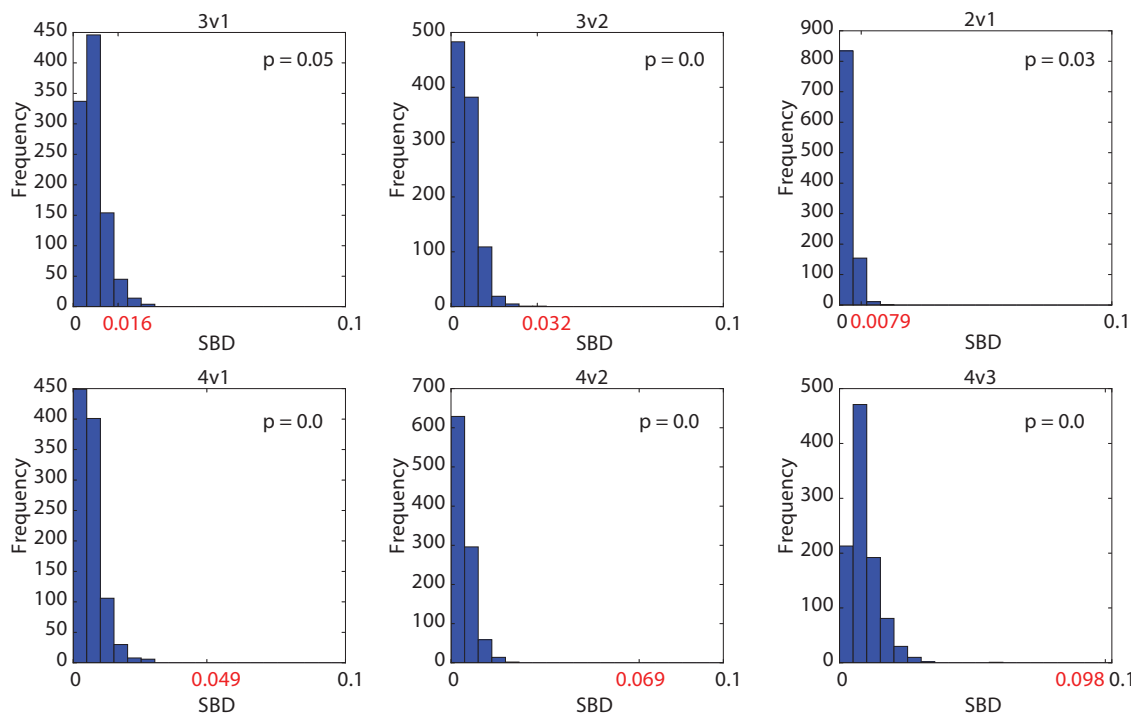


Figure S7

75 minutes



150 minutes



300 minutes

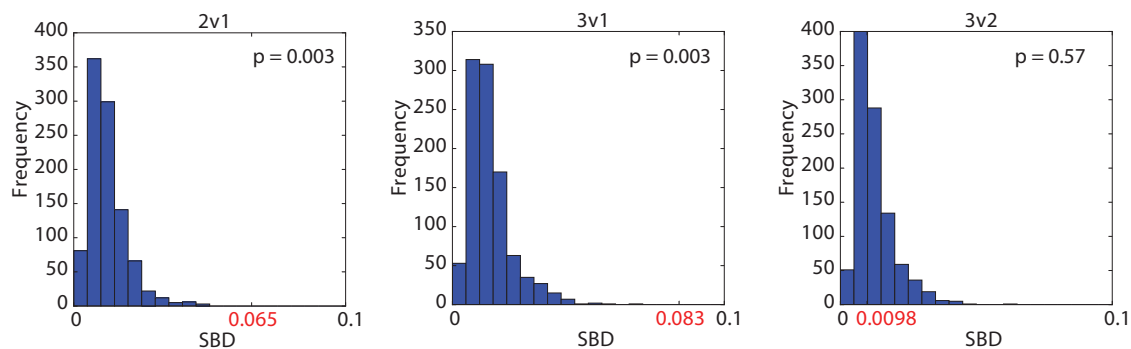


Figure S8

75 minutes

150 minutes

300 minutes

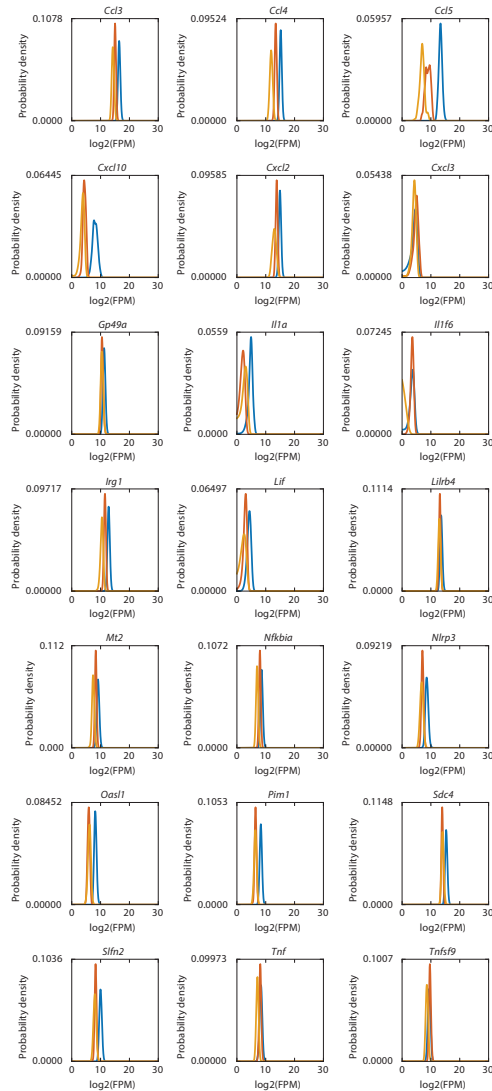
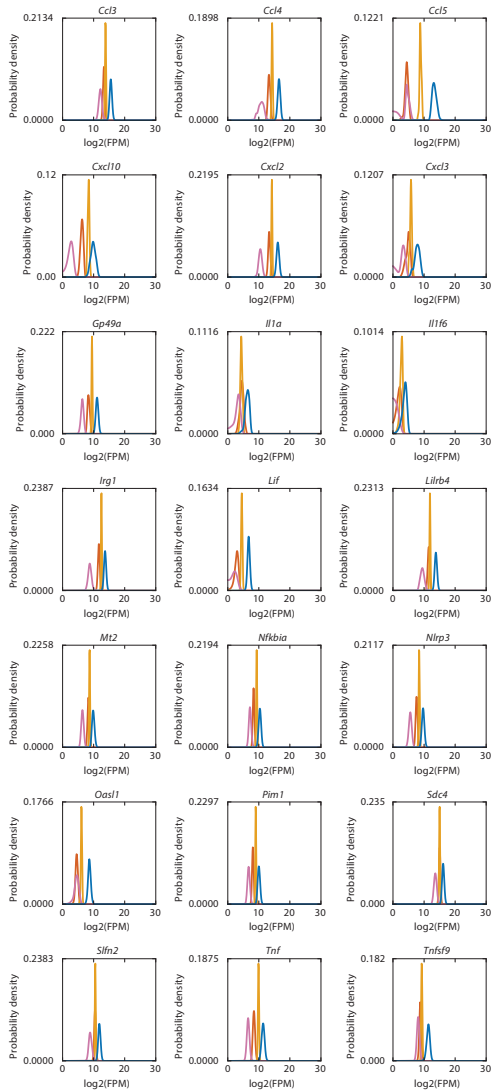
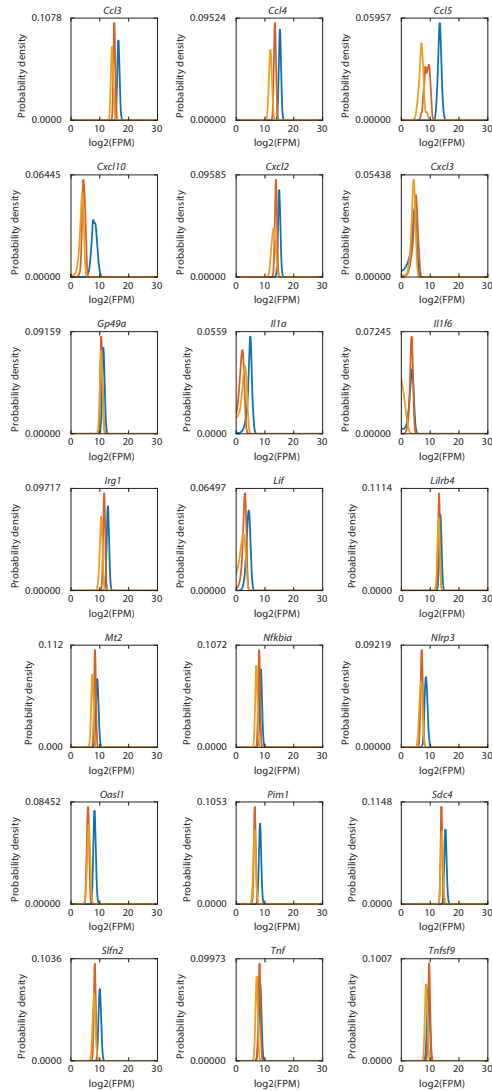


Figure S9

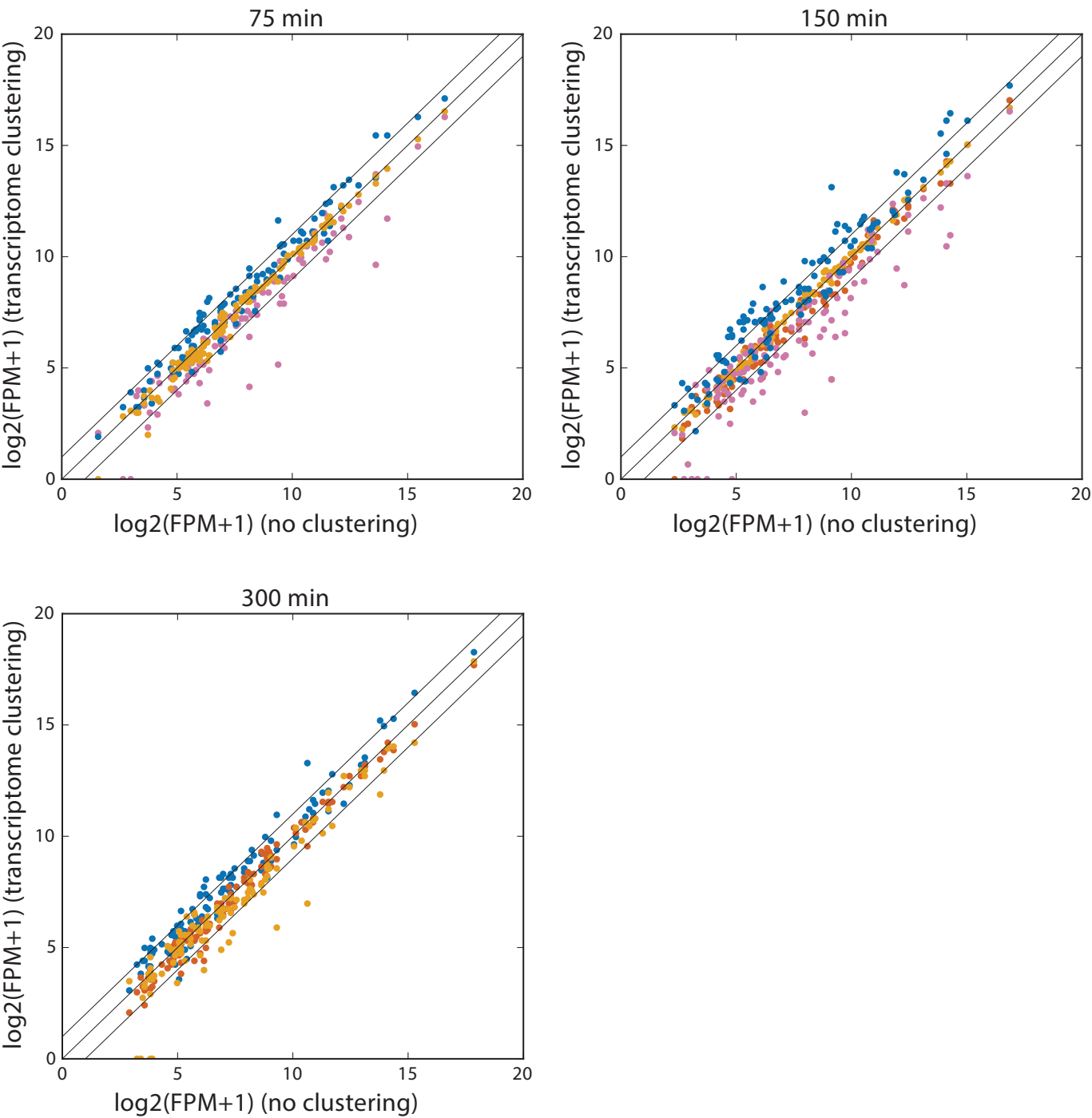


Figure S10

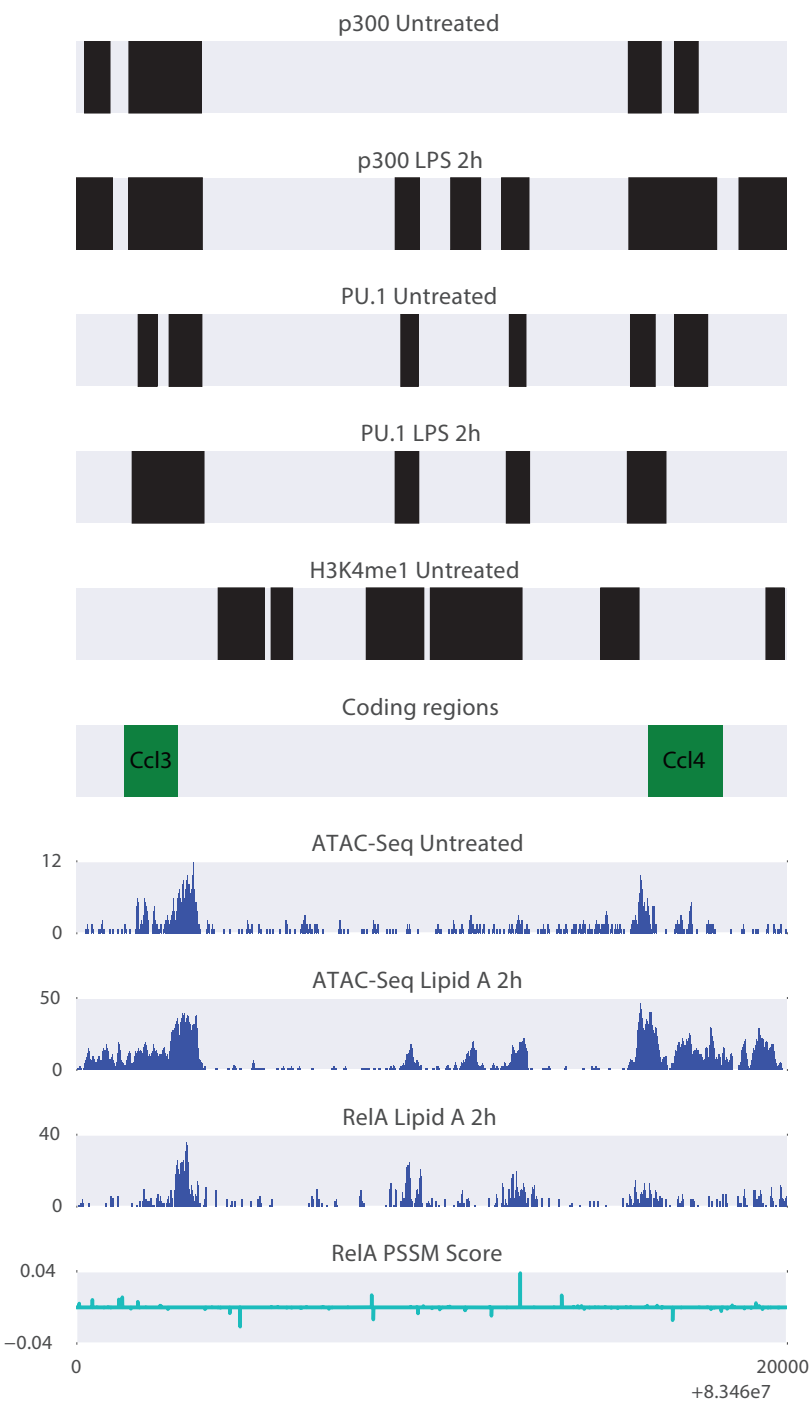
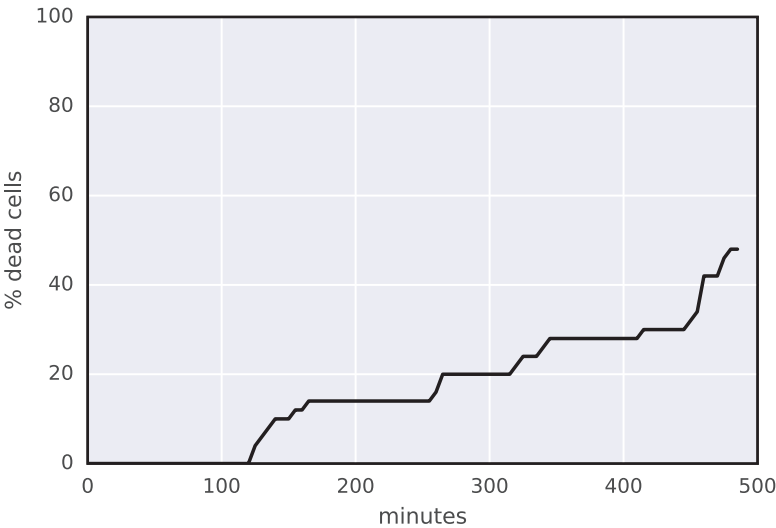
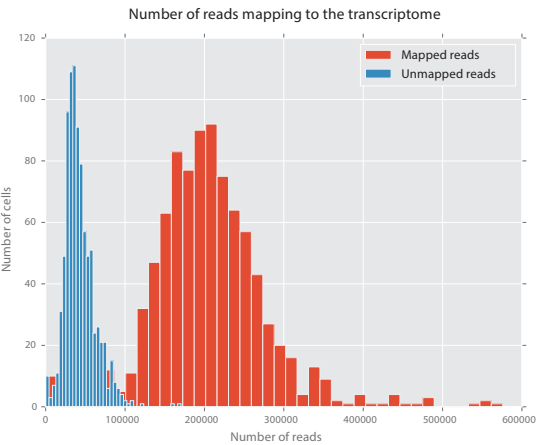


Figure S11

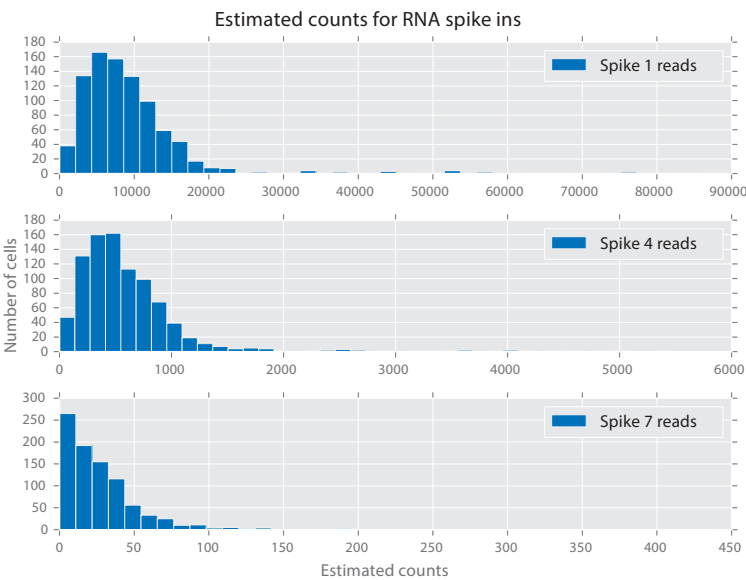
A



B



C



D

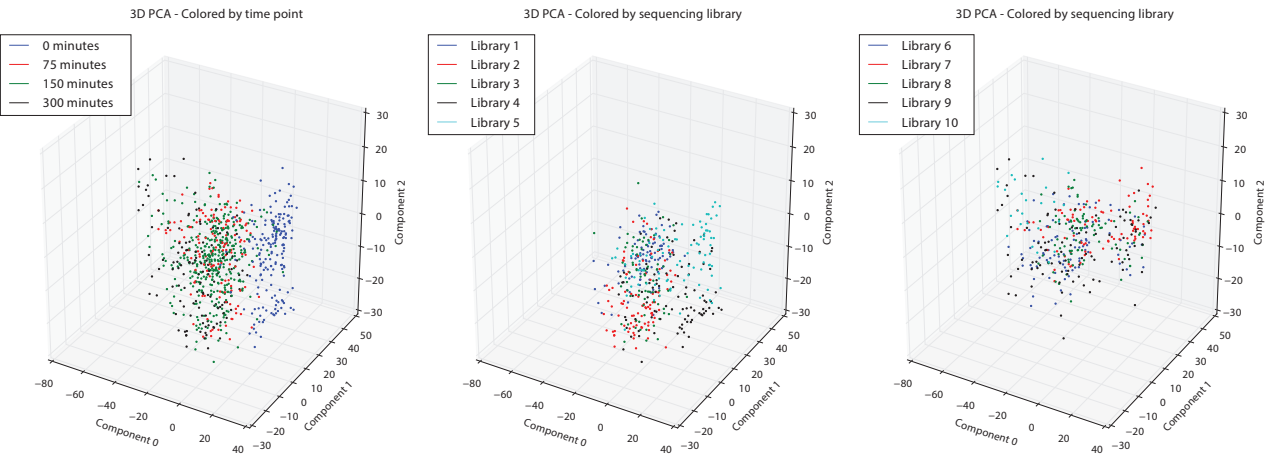


Table S3

75 Minutes

	Dynamics cluster 1 - % Identity	Dynamics cluster 2 - % Identity
Transcriptome cluster 1	7.3%	92.7%
Transcriptome cluster 2	38%	62%
Transcriptome cluster 3	32%	68%

150 Minutes

	Dynamics cluster 1 - % Identity	Dynamics cluster 2 - % Identity	Dynamics cluster 3 - % Identity
Transcriptome cluster 1	17.3%	76.5%	6.2%
Transcriptome cluster 2	21.5%	69.5%	0%
Transcriptome cluster 3	0	92.1%	7.9%
Transcriptome cluster 4	32.7%	40.8%	26.5%

300 Minutes

	Dynamics cluster 1 - % Identity	Dynamics cluster 2 - % Identity	Dynamics cluster 3 - % Identity
Transcriptome cluster 1	14.7%	32.4%	52.9%
Transcriptome cluster 2	8%	60%	32%
Transcriptome cluster 3	2.5%	67.5%	30%

Supplemental Figure Legends

Figure S1, Related to Figure 2.

(A) NF- κ B dynamics with time series clustering for all cells imaged in the C1 IFC. The NF- κ B dynamics are visualized using heatmaps - each row represents one cell while the color represents the nuclear localization of NF- κ B (red - inside the nucleus, white - outside the nucleus). Time series clustering was performed as described using the shape-based distance and hierarchical clustering. Dynamics for the 75 minute, 150 minute, and 300 minute time points are shown.

(B) Representative dynamics for each subpopulation identified by time series clustering at 75 minutes, 150 minutes, and 300 minutes. The algorithm outlined by Paparrizos et al. was used to merge the dynamics of each subpopulation into representative traces (Paparrizos, 2015). By comparing the representative traces across time points, we were able to link the subpopulations found at 75 and 150 minutes with those found at 300 minutes. This linking allows us to follow these subpopulations and their transcriptomes over time. At the 75 minutes timepoint subpopulation 1 and subpopulation 3 dynamics can't be distinguished, as shown by the overlap in their representative traces.

(C) Single-cell gene expression for each time point. For each cell, the FPM's of every gene in our curated list are displayed in a heatmap. Cells are grouped according to the time series clustering approach described earlier.

Figure S2, Related to Figure 2.

Subpopulation transcriptome dynamics for every gene considered in this study. The top 100 differentially expressed genes as determined by SCDE (in comparison to unstimulated cells) at each time point were considered for analysis. Genes with greater than 90% dropout (that is, over 90% of cells having an FPM

of 0) were removed from consideration. A total of 113 genes were identified using this approach. Shaded areas represent 68% confidence intervals.

Figure S3, Related to Figure 3.

(A) Transcript dynamics for all FISH probes. The NF- κ B activity in single cells was monitored with live cell imaging. After 75, 150, or 300 minutes, imaging was halted and the cells were hybridized with probes for *Tnfaip3*, *Ccl4*, *Nfkb1a*, *Tnfsf9*, *Cxcl2*, *Ccl5*, *Nfkbiz*, and *Atf3* using the single-cell RNA FISH protocol. Each cell was then assigned to a subpopulation as described earlier. The median cellular fluorescence within each population is plotted over time. Shaded areas represent the standard error in the median, which was computed using the asymptotic formula for normally distributed data,

$$\text{standard error of median} = 1.253 \times \text{standard error of the mean}.$$

(B) Single cell NF- κ B nuclear localization dynamics for RNA FISH.

Figure S4, Related to Figure 3.

A bootstrapping analysis of differences in gene expression after clustering cells by NF- κ B dynamics. To determine whether the differences in gene expression that we observe between subpopulations after clustering by NF- κ B dynamics could arise from random clustering, we performed a nonparametric bootstrapping analysis of several representative genes (*Ccl3*, *Ccl5*, *Saa3*, *Il1f9*, and *Prdx1*) at the 300 minute time point. This was done by iteratively shuffling the subpopulation labels and then using the SCDE R package to compute the fold change difference in gene expression between each subpopulation. This process was repeated 1000 times, after which we constructed a histogram of the fold changes for each comparison. We used this histogram to compute p-values for our observed fold changes. We found that the gene expression difference between subpopulations that we observe for *Ccl3* and *Ccl5* are highly

unlikely to occur by randomly clustering the cells. We also found that for *Saa3* and *Il1f9*, the gene expression differences between the recurrent dynamics subpopulation and the others were unlikely to occur by random clustering, but that this was not statistically significant using the traditional p-value of 0.05 criterion. We attribute this to the small size of the subpopulation (10 cells).

Figure S5, Related to Figure 3.

(A) The dependence of NF- κ B nuclear localization dynamics on cellular density. RAW 264.7 cells expressing p65-Clover were plated in wells of a 96-well plate at the density indicated, stimulated with LPS and imaged. NF- κ B dynamics were examined in single cells, and the cells were then assigned a subpopulation as described in the Experimental procedures section.

(B) Transcript dynamics upon perturbation of NF- κ B dynamics using Leptomycin B (LMB). The NF- κ B activity in single cells was monitored with live cell imaging in the presence or absence of 18nM LMB. After 75, 150, or 300 minutes, imaging was halted and the cells were hybridized with probes for *Ccl5*, *Nfkb1a*, *Ccl4*, and *Tnfaip2* using the single-cell RNA FISH protocol. Each cell was then assigned to a subpopulation as described earlier. Cells exposed to LMB retained NF- κ B in the nucleus for the duration of the experiment and were assigned to a distinct subpopulation. Transcript dynamics for FISH probes are shown. The median cellular fluorescence within each population is plotted over time. Shaded areas represent the standard error in the median, which was computed using the asymptotic formula for normally distributed data. In these experiments cells belonging to subpopulation 3 were not recovered at sufficient numbers, apart from the *Tnfaip2* experiment.

(C) Single cell NF- κ B nuclear localization dynamics for perturbation experiments with brefeldin A/sTNFR_{II}.

Figure S6. NF- κ B dynamics and gene expression for all time points after clustering cells by their single cell transcriptomes, Related to Figure 4.

(A) NF- κ B dynamics after clustering cells by their transcriptomes for 75, 150, and 300 minute time points.

(B) Representative NF- κ B dynamics for each group identified by transcriptome clustering at each time point.

(C) Single cell gene expression for each time point. Cells are ordered by the transcriptome clustering algorithm.

Figure S7, Related to Figure 4.

A bootstrapping analysis of the differences in representative NF- κ B dynamics after transcriptome clustering. To determine whether the differences between the representative NF- κ B dynamics of the groups determined by transcriptome clustering were statistically significant, we performed a nonparametric bootstrapping analysis. This was done by iteratively shuffling the group labels, computing the representative NF- κ B dynamics, and computing the shape based distance (SBD) between these representative dynamics. This process was repeated 1000 times, after which we constructed a histogram of the shape based distance for each group comparison and then compared it to the actual SBDs we observe from our transcriptome clustering. This analysis was performed for every time point. For almost every comparison, we found that the SBDs between groups that we observe are highly unlikely to appear from random clustering.

Figure S8, Related to Figure 4.

Group-level gene expression at every time point for a set of 21 genes that displayed significant differences in gene expression between groups. From our systems level analysis, we identified 21 genes at the 150 minute time point (*Ccl3*, *Ccl4*, *Ccl5*, *Cxcl10*, *Cxcl2*, *Cxcl3*, *Gp49a*, *Il1a*, *Il1f6*, *Irg1*, *Lif*, *Lilrb4*,

Mt2, *Nfkb1a*, *Nlrp3*, *Oasl1*, *Pim1*, *Sdc4*, *Slfn2*, *Tnf*, and *Tnfsf9*) that displayed > 4 fold differences in gene expression between the highest expressed group and lowest expressed group.

Figure S9, Related to Figure 4.

A systems level view of the link between NF- κ B dynamics and gene expression for every time point. For each gene, we plot the gene expression of each cluster vs the gene expression without clustering. Each data point is colored by the color of the cluster it belongs to. Solid lines represent two fold changes in gene expression.

Figure S10, Related to Figure 4.

A meta-analysis of the epigenetic landscape at the *Ccl3* and *Ccl4* loci. Here, we replot data from (Ghisletti et al., 2010) and (Tong et al., 2016) around the *Ccl3* and *Ccl4* loci after LPS or lipid A treatment. We found a genomic region between *Ccl3* and *Ccl4* that attains the epigenetic signature of a macrophage specific enhancer after stimulation. Chip-Seq data shows that prior to stimulation, this region has PU.1 binding and a H3K4me1 foot print and after stimulation there is evidence of p300 and NF- κ B binding. This region also has a high PSSM score for a RelA binding site. We believe this enhancer may activate both *Ccl3* and *Ccl4*, a model that would explain the high correlation in gene expression that we see between these two genes.

Figure S11, Related to STAR methods.

(A) Cell viability assay. RAW264.7 cells were loaded onto a fibronectin-coated C1 IFC and the cells were stained on chip with the Live/Dead Viability/Cytotoxicity kit according to standard Fluidigm C1 protocols. Cells were imaged at 5min intervals for 8 hours and cell death recorded based on positive

staining with ethidium bromide. The percent cell death is plotted over the course of 8 hours. 50 cells were assayed for this experiment.

(B) Histogram of the number of mapped and unmapped fragments for each cell. Cells with fewer than 200,000 mapped reads, more than 30% of mapped reads originating from the RNA spike-ins, or more than 50% of reads unmapped were excluded from further analysis.

(C) Histogram of the number of fragments mapping to each of the three RNA spike-ins for each cell. Three different spike-ins, each added at progressively lower concentrations, were added to each cell. The spike-ins were used to identify low quality cDNA libraries.

(D) 3D PCA of the single-cell RNA sequencing library. We colored the data points either by time point or by library number to see whether the data would cluster. As expected, the data forms well-defined clusters when colored by time point. The data does not cluster well when colored by library number, demonstrating that strong batch effects are not present in our sequencing data.

Table S1, Single_cell_dynamics.csv, Related to Figure 2.

This file contains the processed NF- κ B dynamics for all of the cells measured on the C1 platform.

Table S2, Single_cell_transcriptomes.csv, Related to Figure 2.

This file contains estimates of transcript abundance for the 113 genes considered in this study for all of the cells measured on the C1 platform.

Table S3, Related to Figure 4.

This table provides the quantitative composition of the clusters identified by transcriptional clustering in terms of the clusters defined by NF- κ B dynamics clustering at each time point.

Data S1, Related to STAR Methods.

Custom Fluidigm C1 scripts for cell loading and cell stimulation.